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MECHANISMS OF CELL INJURY WITH HEPATOTOXIC CHEMICALS

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Toxic chemicals can be broadly divided into two classes. The first requires metabolism by the target cells, and the cell killing is dependent on this metabolism. The second group does not require metabolism by the target cells. Compounds that require metabolism can be further subdivided into those that are metabolized by the cytochrome P-450 mixed function oxidases and those that are not. Much of the work on the mechanisms of action of toxic chemicals has dealt with compounds that are metabolized by mixed function oxidation, and the present discussion will focus principally on the results of these studies.

There are three consequences of the metabolism of toxic chemicals by mixed function oxidases that may be responsible for the genesis of membrane alterations that may in turn relate to the genesis of lethal cell injury: 1) the formation of free radicals, 2) the formation of electrophilic products, and 3) the generation of activated oxygen species.

Carbon Tetrachloride-Induced Liver Necrosis

Carbon tetrachloride is the best known example of a chemical whose toxicity is believed to be the consequence of the formation of free radicals. CCl_4 is metabolized by a particular ferrous cytochrome P-450, and its toxicity is dependent on this metabolism (Recknagel and Glende, 1973). The initial event seems to be homolytic cleavage of a carbon-chlorine bond with formation of a chloride ion and the trichlormethyl radical (CCl_3). This radical has been detected by spin-trapping (Lai et al., 1979; Poyer et al., 1978; Poyer et al., 1980). The trichlormethyl radical is highly reactive, and there is considerable controversy over which of its several fates is related to the liver necrosis. The trichlormethyl radical may react with O_2 to form trichloromethyl dioxide, then reduced to trichloromethanol which rapidly decomposes to phosgene ($COCl_2$) by dehydrohalogenation. Phosgene has

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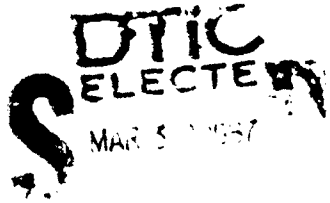

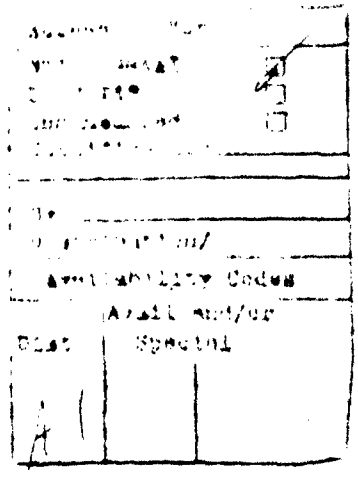

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been detected (Kubic and Anders, 1980; Shah et al., 1979), but the yield is low, and it is questionable whether phosgene generation is toxicologically significant (Waller and Recknagel, 1982). The trichlormethyl radical can also covalently bind to microsomal membrane lipids and proteins or abstract a hydrogen atom from unsaturated lipid to produce chloroform (CCl_3H) and initiate oxygen consuming lipid peroxidation. These events appear to be confined to regions of the endoplasmic reticulum at or near the cytochrome P-450 locus.

Recent studies of CCl_4 liver injury have concerned the way the initial consequences of CCl_4 metabolism are linked to subsequent cell membrane breakdown and death of the cell. Considerable attention has been focused on the role of the peroxidation of the microsomal membrane lipids. Lipid peroxidation generates a variety of more or less toxic products which can in principle migrate from membrane sites near cytochrome P-450 to other parts of the cell. Dialysates of peroxidizing microsomes lysed red cells from vitamin E deficient rats (Benedetti et al., 1977). The active fraction contained 4-hydroxyalkenals, of which 4-hydroxynonenal was quantitatively the most significant (Benedetti et al., 1980). Biogenic 4-hydroxyalkenals can inhibit liver microsomal glucose-6-phosphatase, kill isolated hepatocytes, lyse red blood cells, and inhibit protein synthesis in a rabbit reticulocyte lysate (Benedetti et al., 1980; Benedetti et al., 1981). The identification of 4-hydroxyalkenals as the presumed secondary toxicological messengers in CCl_4 liver injury seemed to offer a satisfactory explanation of the sequence of events linking lipid peroxidation with the death of the cell.

Doubts about the role of 4-hydroxyalkenals, however, have been raised (Recknagel, 1983). The reported LD_{50} for the cytopathologic effects of biogenic 4-hydroxyalkenals and synthetic 4-hydroxynonenal range from 0.1 to 0.5 mM (Benedetti et al., 1980; Benedetti et al., 1981). It can be questioned whether the restricted lipid peroxidation associated with CCl_4 metabolism can generate such large amounts of 4-hydroxyalkenals that seem to be necessary for these compounds to act as the putative vector. Furthermore, 4-hydroxyalkenals readily react with -SH groups of low molecular weight thiols (GSH, etc.) and of proteins. The GSH content of the liver falls minimally (Pohl et al., 1981) or not at all (Docks and Krishna, 1976) after CCl_4 intoxication. The concentration of GSH in the liver should be more than adequate to react with potentially toxic 4-hydroxyalkenals arising from CCl_4-dependent lipid peroxidation. These considerations generate some scepticism that the products of lipid peroxidation can link this process to pathological consequences at distant cellular sites (Recknagel, 1983).

Are the mechanisms of liver cell injury by CCl_4 relevant to the action of other hepatotoxic chemicals? The central event in CCl_4 metabolism is the cleavage of a carbon-chlorine bond. Although this is catalyzed by a cytochrome P-450 mono-oxygenase, there is no enzymatic incorporation of oxygen into the substrate. There are a few other hepatotoxic polyhaloalkanes that seem to be metabolized in a manner similar to CCl_4. The ease of carbon-halogen bond reduction increases with the atomic number of the halogen atom. Bromotrichloromethane is 30- to 40-fold more hepatotoxic than CCl_4 (Sipes et al., 1977), and its ability to induce lipid peroxidation is 200 times greater (Slater and Sawyer, 1971). It is presumed that these effects result from the lower bond dissociation energy of the C-Br bond relative to the C-Cl bond, which facilitates an increased rate of trichloromethyl free radical production.

Halothane ($CF_3CHClBr$) is another hepatotoxin whose metabolism may resemble that of CCl_4. Reductive C-Br bond rupture is suspected to produce the 1,1,-trifluoro-2-chlorethyl radical, which abstracts hydrogen from a lipid to generate 1,1,1-trifluoro-2-chloroethane (Ahr et al., 1982; Brown and Sipes, 1977; Kubic and Anders, 1981). Like carbon tetrachloride, reductive metabolism of halothane induces lipid peroxidation (Sipes et al., 1980).

The Role of Covalent Binding in Toxic Cell Death

The second mechanism whereby metabolism of toxic chemicals by the mixed function oxidases may damage cellular membranes is the covalent binding of electrophilic products to macromolecules. Cellular injury mediated by the interaction of chemically reactive metabolites with macromolecules has been of xenobiotic chemicals and drugs (Zimmerman, 1978). Much of the evidence implicating the covalent binding of reactive metabolites to the subsequent cell death is based on studies of bromobenzene- and acetaminophen-induced liver necrosis in rodents.

Bromobenzene is an environmental contaminant that can cause hepatic, renal, and pulmonary necrosis. Cytochrome P-450-dependent metabolism is necessary for the expression of these biological effects. Bromophenol isomers are the major products formed, which vary (ortho:meta:para bromophenol) depending on the enzyme source and pretreatment. The phenols result from the reaction of an initial product, an arene oxide, with water (Selander et al., 1975). Formation of the 3,4-epoxide is believed to lead to macromolecular binding and tissue necrosis (Lau et al., 1979; Lau and Zannoni, 1979; Lau et al., 1980; Zampaglione et al., 1973).

The evidence to support such a role for covalent binding is largely circumstantial and based on the persistent correlation between the extent of binding and the severity of the accompanying liver cell necrosis (Brodie et al., 1971; Gillette et al., 1974; Jollow et al., 1974; Reid et al., 1971; Zampaglione et al., 1973). Bromobenzene-induced liver necrosis is predominantly centrilobular, and the covalent binding of [^{14}C]bromobenzene is predominantly centrilobular. Induction of mixed function oxidase activity with phenobarbital increases the ability of the hepatocytes to metabolize bromobenzene, increases the covalent binding of [^{14}]bromobenzene, and increases the extent of liver necrosis. Conversely, inhibition of mixed function oxidase activity with SKF-525A reduces bromobenzene metabolism, reduces the covalent binding, and reduces the extent of liver necrosis.

Despite the strength of the correlation, there is little direct evidence to substantiate the hypothesis that covalent binding to macromolecules can produce cell lethal injury. In particular, the molecular targets which interact with reactive metabolites and lead to cell death have not been identified. In addition, the functional consequences of such interactions between the chemical toxin and key cellular targets have rarely been considered. Nevertheless, covalent binding remains a widely accepted hypothesis. It is frequently used as an endpoint in the study of the metabolism of toxic chemicals to either explain the mechanism of action of a known toxin or to predict the biological activity of a suspected toxin. The covalent binding hypothesis remains a dominant organizing principle in molecular toxicology because of the existence of only limited data inconsistent with it.

Acetaminophen is another chemical whose toxicity has been closely correlated with the covalent binding of its metabolites to macromolecules (Davis et al., 1974; Jollow et al., 1973; Mitchell et al., 1973; Potter et al., 1974). There have been a few reports, however, of a negative correlation between covalent binding and liver necrosis with acetaminophen. Pretreatment of mice with a-mercaptopropionyl glycine gave significant protection against acetaminophen-induced hepatic necrosis without appreciably affecting the amount of drug covalently bound to hepatic proteins (Lambadarios et al., 1977). Comparable observations were made with N-acetylcysteine (Gerber et al., 1977). In a more recent report, acetaminophen toxicity was studied in isolated rat hepatocytes prepared from animals pretreated with either phenobarbital or methylcholanthrene (Casini et al., unpublished results; Devalia et al., 1982). Methylcholanthrene renders rodents more susceptible to acetaminophen-induced liver cell injury than does phenobarbital treatment (Potter et al., 1974). Exposure of

phenobarbital-induced hepatocytes to 10 mM acetaminophen caused a loss of intracellular GSH and a progressive leakage of isocitrate dehydrogenase (Devalia et al., 1982). Treatment with 3-O-methyl-(+)catechin prevented the leakage of ICD without affecting the amount of material covalently bound to cellular protein or the GSH loss. In methylcholanthrene hepatocytes, recent data also suggested a dissociation of covalent binding from the cell killing by acetaminophen (Casini et al., unpublished results). The time course of the binding preceded that of the cell death, as reported with bromobenzene (Casini et al., 1982). Addition of either DPPD or promethazine to the culture medium reduced by only 50% the extent of the covalent binding while completely preventing cell killing. If promethazine was given at increasing times up to 3 hours after the [^{3}H]acetaminophen, 70-80% as much covalent binding as developed without promethazine could occur with little or no cell death (Casini et al., 1982).

The Role of Activated Oxygen in the Toxicity of Mixed Function Oxidase Substrates

The third mechanism by which mixed function oxidase-dependent biotransformation may produce liver cell injury is by the production of activated oxygen species. A branch point exists in the P-450 catalytic cycle at the introduction of the second electron (White and Coon, 1980). Active turnover by the P-450 system involves production of hydrogen peroxide formed from the dismutation of superoxide dismutase. As much as 55% of consumed oxygen can appear as H_2O_2 in the presence of appropriate substrates. Several recent studies have suggested that the oxidative stress imposed by the formation of O_2- and H_2O_2 can offer an alternative to covalent binding as an explanation of the biological activity of many toxic chemicals.

The killing of cultured hepatocytes by the aryl halides chloro-, bromo-, and iodobenzene is accompanied by the peroxidation of cellular lipids as shown by the accumulation of malondialdehyde and by the appearance of conjugated dienes in cellular phospholipids (Casini et al., 1982; Casini et al., unpublished results). DPPD as well as other antioxidants reduced this lipid peroxidation in proportion to the reduction of the cell death.

The simplest explanation of these data would be the peroxidation of dead cells. If lipid peroxidation, however, were simply occurring in dead cells, then antioxidants should have prevented it without having any effect on the viability of the cells. When hepatocytes were killed by mechanisms that do not involve peroxidation of cellular lipids, delayed accumulation of

malondialdehyde occurred (Casini et al., 1982). Antioxidants such as DPPD had no effect on the cell killing. They did, however, prevent the peroxidation of lipids, which was clearly occurring in dead cells. In contrast, the antioxidants DPPD and promethazine prevented the cell killing in parallel with their ability to prevent the peroxidation of lipids in the aryl halide-intoxicated hepatocytes (Casini et al., 1982; Casini et al., unpublished results). This would suggest that a more complex interpretation of the lipid peroxidation is needed. More likely the lipid peroxidation must be either causally related to the cell death or must be the product of an undefined event that is responsible, in turn, for the loss of viability. Such an event must also be sensitive to antioxidants.

Lipid peroxidation in aryl halide-intoxicated hepatocytes can be ascribed to the combined effects of the formation of activated oxygen species and the depletion of GSH stores. An oxidative stress accompanying the mixed function oxidase-metabolism of an aryl halide would normally be dealt with by mechanisms dependent upon GSH. The depletion of GSH as a result of its reaction with the electrophilic epoxides derived from the aryl halide would render the liver cells relatively defenseless against the potentially toxic activated oxygen species with the resultant peroxidative decomposition of cellular phospholipids.

In methylcholanthrene-induced hepatocytes, acetaminophen reduced the GSH levels by 85% within 2 hours, and lipid peroxidation accompanied the loss of viability (Casini et al., unpublished results). The antioxidants DPPD and promethazine again prevented both the lipid peroxidation and the death of the cells. Lipid peroxidation has also been reported with acetaminophen toxicity in intact animals (Wendel et al., 1979; Wendel and Feuerstein, 1981; Wendel et al., 1982). Feeding male mice sucrose decreases glutathione and increases the acetaminophen-induced lipid peroxidation and liver necrosis. Inducers of drug metabolizing enzymes increased the evidence of lipid peroxidation and liver necrosis in parallel.

Recently it has been reported that acetaminophen is oxidized in vitro by cytochrome P-450 to produce an acetaminophen free radical and hydrogen peroxide (Rosen et al., 1983). It was suggested that cytochrome P-450 initiates a one-electron oxidation of acetaminophen to give a free radical. Transfer of an electron from this radical to oxygen would produce superoxide and N-acetyl-p-benzoquinone imine. In addition it was shown that microsomal lipid peroxidation was markedly stimulated in the presence of acetaminophen (Rosen et al., 1983).

By way of summary of the above discussion, three mechanisms have been considered to account for the membrane damage that results from the metabolism of toxic chemicals by mixed function oxidases. Free radical formation is best known with the metabolism of a few polyhalomethanes by the enzyme catalyzed addition of a single electron to a carbon-halogen bond followed by its homolytic cleavage. The highly reactive free radicals thus formed can covalently bind to proteins and lipids or initiate lipid peroxidation by hydrogen abstraction from unsaturated fatty acids. Both of these effects seem to occur in close proximity to cytochrome P-450, and it is still unclear how the initial membrane alterations lead to the later loss of plasma membrane integrity and the death of the cell. More commonly, metabolism occurs by oxygen insertion and the formation of an electrophilic product. These can react with cellular nucleophiles including proteins and lipids. Such covalent binding to cellular macromolecules has been widely held to be the major mechanism coupling metabolism to the biological consequences of most chemical toxins. There is little direct evidence, however, to support such a relationship, and recent studies with cultured hepatocytes have weakened somewhat the hitherto persistent correlation between covalent binding and liver necrosis. Finally, the formation of activated oxygen species, O_2- and H_2O_2, accompanies the metabolism of xenobiotics as well as other mixed function oxidase substrates. The loss of GSH as a result of its conjugation with the electrophilic products of toxin metabolism may weaken cellular defenses to such a point that the activated oxygen species become toxic.

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